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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The ultimate goal of the research project is to reconstruct the 3-dimensional quaternary structure of the IGF-II/isoform A (IR-A) of the insulin receptor by cryo scanning transmission electronmicroscopy (cryoSTEM) using the electron-dense gold-labeled IGF-II to visualize the complex. During the first year of the grant support we succeeded in preparing in micro-scale gold-labeled IGF-II by reacting IGF-II with sulfo-NHS-Nanogold. The gold labeling occurred at the N-terminal alanine of IGF-II. The gold-IGF-II, like native IGF-II, activated the autophosphorylation of the isoform A (IR-A) but not the isoform B (IR-B) of the insulin receptor. We began this second year of grant support to scale up the preparation of the gold-IGF-II. We have now prepared a sufficient quantity (approx. 100 µg) of gold-IGF-II which is biologically as active as IGF-II in activating the autophosphorylation of IR-A. In preparation for the formation of the IGF-II/IR-A complex we have begun the large-scale preparation of IR-A protein from cultures of transfectant cells expressing this receptor protein.

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Introduction

Insulin-like growth factor I and II (IGF-I and IGF-II) are related peptide hormones sharing a high degree of sequence and structural homology with insulin. While insulin acts mainly to regulate glucose uptake and cellular metabolism, IGFs play a major regulatory role in growth and proliferation in most cells and tissues. It is generally believed that both IGFs exert their actions through the receptor for IGF-I (IGF-I-R). IGF-I-R is highly homologous to the transmembrane insulin receptor (IR) (1). Human IR has two isoforms (IR-A and IR-B) resulting from the alternate splicing of a small exon (exon 11) encoding 12 aminoacids (residues 718-729) at the carboxyl end of the receptor α-subunit. IR-A is the shorter isoform. It was reported recently that IGF-II binds to IR-A, but not IR-B, with an affinity close to that of insulin (2), and of particular significance is that binding of IGF-II to IR-A leads to predominantly mitogenic responses. Furthermore, it was observed that the relative abundance of IR-A was significantly higher in breast and colon cancer tissues than in normal tissues, and that receptor autophosphorylation induced by IGF-II reflected the relative abundance of IR-A. These novel observations show that, as a growth factor, IGF-II can interact effectively with IR-A, and could be biologically relevant and significant in cancers where IGF-II is locally produced and IR-A is the predominant isoform of IR. This research project focuses on the question of what may be the structural basis for the preferential binding of IGF-II to IR-A over IR-B. The aims of the research are to obtain the quaternary 3-D structure of the complex of IGF-II and IR-A, to locate the binding site of IGF-II in the structure, and to compare the location with that of the insulin-binding site. Low-dose (6 electrons/$Å^2$), and low-temperature (-150°C) dark field scanning transmission electron microscopy (STEM) will be used to obtain the quaternary 3-D structure of the complex formed between IGF-II and IR-A. The P.I. and colleagues have successfully used this method to obtain the 3-D structure of the insulin receptor through the use of insulin labeled with Nanogold (3) as an electron-dense ligand to obtain images of the insulin/receptor complex. The 3-D structure thus obtained has made it possible to identify on the IR amino acid residues involved in the binding of insulin (4). The scope of the project includes the preparation of biologically active IGF-II labeled with Nanogold, preparation and purification of IR-A and IR-B protein, formation of the Nanogold-IGF-II complex with IR-A, and STEM imaging of the complex for 3-D reconstruction.

Body

IGF-II binds to the human insulin receptor isoform A (IR-A) with an affinity equal to that of insulin. While insulin binding to IR-A primarily leads to metabolic responses, IGF-II binding produces mainly mitogenic effects. We postulate that the differential response is a result of IGF-II binding to a site different from but shared with the insulin-binding site on IR-A. In a recent study, using insulin labeled with Nanogold (Nanogold-insulin) we applied the method of cryoSTEM to reconstruct the 3-D quaternary structure of the insulin/IR complex (3), and were able to pinpoint the insulin-binding site on the 3-D structure of the IR (4). Accordingly, in this research project we aim to apply this method to determine the IGF-II-binding site on 3-D structure of the two IR isoforms (IR-A and IR-B), and to compare with the insulin-binding site. The research

project has been planned in a logical sequence: 1) preparation and characterization of Nanogold-IGF-II; 2) preparation and purification of IR-A and IR-B proteins; and 3) determination of the 3-D quaternary structure of each of the ligand-receptor complexes.

In accordance to the Statement of Work, the research project has been scheduled as follows: **Year 1-2**: preparation and characterization of Nanogold-IGF-II, and preparation and purification of IR-A and IR-B; **Year 2-3:** determination of the quaternary 3-D structure of the IGF-II-IR-A and IGF-II-IR-B complex. As a mid-term report, research accomplishments during **Year 1-2** are described below.

1) Preparation and Characterization of Nanogold-IGF-II

To recapitulate briefly the work done during Year 1 which had been reported in the Annual Report (1 May 00 – 1 May 01), we accomplished the followings: i) establishing the optimal conditions (pH, temperature, time, and molar ratios of reactants) to label IGF-II with mono-NHS-Nanogold (Nanoprobes Inc., Stoneybrook, NY); ii) separation and purification of the reaction products by size-exclusion HPLC at neutral pH; iii) establishing by peptide sequencing and mass spectrometry that IGF-II was labeled at its N-terminal alanine with a gold cluster; and iv) showing that the Nanogold-IGF-II obtained was biologically active like native IGF-II when assayed against IR-A and IR-B. The accomplishments in Year 1 had set the stage for the large-scale preparation of Nanogold-IGF-II at the beginning of Year 2.

During the first year we had experienced some lot-to-lot variability in reactivity of the reagent mono-NHS-Nanogold. Therefore we thought it prudent to test out the reagent in small lots from the supplier (Nanoprobes, Inc.) before ordering a large lot of the reagent for the large-scale reaction. Unexpectedly and most disturbing beginning in late April 2001 using different lots of reagent obtained from the supplier we were unable to label IGF-II as we had done before. We spent nearly 3 months rechecking our reaction conditions without success until the supplier, after much probing from us, owed up to us in July 2001 that it has altered the original formulation of the reagent which is now highly positively charged. Because the reagent is proprietary to the supplier, we have been unable to learn exactly what other alterations may have been made to the original reagent such that it no longer reacts with IGF-II. Between July and September 2001 we tried different reaction conditions with the newly formulated reagent in an attempt to label IGF-II without success. After much discussion with the supplier in an attempt to get to the bottom of the problem, we eventually persuaded the supplier to custom prepare one lot (Lot# JS8061A) of the reagent of the original formulation. A small sample of which was tested and found to be reactive with IGF-II as originally observed. However, the reactivity of this reagent was still less than what we had experienced previously in that the conversion of IGF-II to the gold-labeled derivative was not 100%. Nonetheless, we felt that we should be able to produce a sufficient quantity of gold-labeled IGF-II for forming the complex. We wish to note that altering the original formulation of the reagent by the supplier has been the major and a serious problem that has caused a setback of nearly 5 months to the progress of the research project that had proceeded well during the first year.

The large-scale preparation of Nanogold-IGF-II using the customized lot of mono-NHS-Nanogold was carried out in multiples of 30 nmoles of the reagent as described below.

Mono-NHS-Nanogold (30 nmoles) dissolved in 50 μL DMSO was added to 300 μg of IGF-II dissolved in 300 μL of sodium phosphate buffer (0.1 M, pH 8.5). The reaction mixture was incubated at 30° C for 43 hours in an air shaker (250-280 strokes/min.). The reaction was stopped with 15 μL of ethanolamine, and then lyophilized. An aliquot of the reaction mixture was analyzed by acid-urea polyacrylamide gel electrophoresis that separates modified IGF-II from native IGF-II by charge. When we were establishing the reaction conditions in small-scale reaction in Year 1, we obtained two gold-labeled products of IGF-II with slightly different electrophoretic mobility, both being biologically active. In contrast, in this large-scale reaction only one band, or one gold-labeled product was obtained. **Figure 1** shows that after 43 hr of incubation the ratio of gold-labeled IGF-II (appeared as a stained band with a slower electrophoretic mobility than native IGF-II) to unreacted IGF-II in the reaction mixture was about 1:1 in all three separate large-scale preparations. Aliquots of the reaction mixtures were also separated by electrophoresis in 10-20% Tricine polyacrylamide gel that separates peptides of low molecular weight by size. The gel was stained for the presence of gold with a silver enhancement kit from Nanoprobes, Inc. A stained band with a molecular mass expected of Nanogold-IGF-II was detected (data not shown), confirming the presence of the gold cluster on the labeled IGF-II. This was additional evidence to the analysis by mass spectrometry (done in Year 1) for the presence of gold.

Each large-scale reaction mixture was separated by HPLC through a size exclusion column (BioSil SEC I-125-5, 300 mm x 7.8 mm) developed with 50 mM sodium phosphate buffer, pH 7.6, at a flowrate of 0.5 mL/min. Fractions of 0.5 mL were collected. Aliquots of fractions were analyzed by acid-urea polyacrylamide gel electrophoresis. **Figure 2** shows the typical separation of Nanogold-IGF-II from unreacted IGF-II and side products of the reactions. Fractions containing Nanogold-IGF-II but free from unreacted IGF-II were pooled. Fractions containing both Nanogold-IGF-II and unreacted IGF-II were pooled for re-chromatography to obtain additional Nanogold-IGF-II free from unreacted IGF-II. Fractions containing Nanogold-IGF-II free from unreacted IGF-II were pooled and lyophilized in aliquots. Amount of Nanogold in each aliquots was determined by amino acid analysis. As expected, peptide sequence analysis showed that the N-terminal alanine was blocked. Approximately 100 μg of Nanogold-IGF-II were obtained, sufficient for the large-scale preparation of Nanogold-IGF-II/IR-A and IR-B complexes for STEM reconstruction.

2) Preparation of pure IR-A and IR-B receptors

R- cells are mouse fibroblasts with an IGF-I R knockout and have very low numbers of mouse IR. These cells were transfected with either human IR-A or IR-B. (R-) IR-A and (R-) IR-B cells were grown in 150 cm^2 flasks until 90% confluent. Cells were solubilized in 10 mL 50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton-X100, 1 mM PMSF for 1 hour at 4°C. Next, lysates were centrifuged at 100,000 x g for 60 minutes, and frozen at -80°C until purification. MA51 column, an anti-insulin receptor (human) antibody coupled to Affigel 15, was equilibrated with 50 mM Hepes, pH 7.6, 150 mM

NaCl, 0.1% Triton-X100, 1 mM PMSF (WGA buffer). Lysates from cells were passed over the MA51 column and pass-through was collected. The pass-through was recycled 3 times. The insulin receptors were allowed to bind overnight at 4°C. Pass-though fractions were collected and the column was washed with 50 ml of WGA buffer. Next, column was washed with 20 mL 50 mM Hepes, pH 7.6, 1 M NaCl, 0.1% Triton-X100, 1 mM PMSF. Insulin receptors were eluted with 2.5 M $MgCl_2$, 120 mM borate, 0.1% Triton-X100, 1 mM PMSF into 180 ml WGA buffer. The eluted insulin receptors were then applied to wheat germ agglutinin agarose column. After washing with WGA buffer, the insulin receptors (IR) were eluted with 50 mM Hepes, pH 7.6, 300 mM N-acetyl-D-glucosamine, 0.1% Triton-X100, 1 mM PMSF. IR content was determined by specific IR ELISA. **Figure 3** shows the SDS-PAGE of purified IR-A after reduction to its 130-kDa α subunit and 95-kDa β subunit. The differential response of purified IR-A and IR-B to IGF-II and insulin in receptor autophosphorylation is shown in **Figure 4,** confirming that, like insulin, IGF-II activates IR-A, but unlike insulin it does not activate IR-B.

3) Receptor autophosphorylation ELISA

96-well plates were coated with 2 μg/mL of MA-20, a monoclonal antibody that recognizes and captures both IR-A and IR-B in their native and tyrosine phosphorylated states. After blocking with SuperBlock (Pierce Chemical), cell lysates containing 1 ng IR (IR-A or IR-B) was applied to each well and allowed to bind overnight at 4°C. Next, wells were washed with TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20). To read out the tyrosine phosphorylated IR, 100 μL goat anti-PY coupled to horse-radish peroxidase (1:2000) was added for 2 hours at 22°C. After washing with TBST, signal was developed by using TMB substrate.

Purified Nanogold-IGF-II obtained from the large-scale preparation was assayed for its ability to activate receptor autophosphorylation of IR-A in comparison with insulin and IGF-I. Results shown in **Figure 5** confirm that the gold-labeled IGF-II, like insulin, activates IR-A as expected from preliminary results obtained in Year 1 while establishing conditions for reaction and purification.

Key Research Accomplishments

There has been no deviation from the Statement of Work described in the original proposal. In spite of the delay (reported above) caused by the supplier changing the formulation of the mono-NHS-Nanogold, we have accomplished in Year 2 the followings as expected by the Statement of Work:

- Following the conditions worked out in Year 1, we have successfully prepared about 100 μg of purified Nanogold-IGF-II, sufficient for the formation of complex with IR-A and with IR-B for 3-D reconstruction of the complex in Year 3.
- As expected for IGF-II, like insulin, the Nanogold-IGF-II obtained activates IR-A.
- Large-scale (mg) extraction and purification of IR-A and IR-B from transfectant cells have begun for the formation of complex with the Nanogold-IGF-II.

Reportable Outcomes

1. An abstract entitled "IGF-II and Insulin Receptor" by Cecil C. Yip and Ira D. Goldfine has been submitted to the Third DOD Breast Cancer Research Program Era of Hope meeting, September 11-14, 2002.

Conclusions

Based on conditions established in Year 1 of the IDEA grant for the micro-scale (less than 10 μg) preparation and purification of biologically active Nanogold-IGF-II, in this past year we have prepared a sufficient quantity (~ 100 μg) of this gold-labeled IGF-II to be used for the formation of a complex with each of the two human insulin receptor isoforms, IR-A and IR-B. The product has been confirmed to be gold-labeled at the N-terminal alanine of IGF-II, and, like IGF-II and insulin, activates the autophosphorylation of IR-A. Extraction and purification of IR-A and IR-B protein in mg quantities from transfectant cells have begun.

In conclusion, the research project is on target. As we begin Year 3, we will be preparing the complexes between Nanogold-IDF-II and the two isoforms (IR-A and IR-B) of insulin receptor. CryoSTEM will be performed on the complexes to obtain the 3-D reconstruction of their quaternary structure in the third and final year of projct, meeting the objectives and final goal set out in the Statement of Work.

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3. Luo, R. Z-Y., Beniac, D.R., Fernandes, A., Yip, C.C., and Ottensmeyer, F.P., Science **285**: 1077-1080 (1999).
4. Ottensmeyer, F.P., Beniac, D.R., Luo, Robert Z-T., and Yip, C.C., Biochemistry **39**: 12103-12112 (2000).

APPENDICES

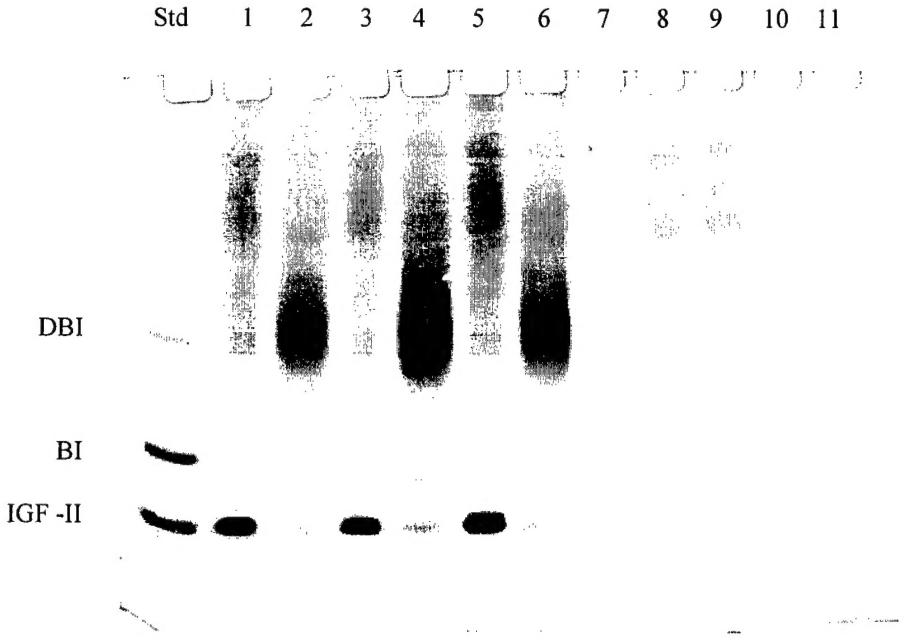

Figure 1 Acid-urea polyacrylamide gel electrophoresis of reaction mixture obtained from 3 separate reactions, each using 300 μg IGF-II and 30 nmoles of mono-NHS-Nanogold, at 29-30° C.

Lane 1, 3, 5: reaction after 2 min.

Lane 2, 4, 5: reaction after 43 hours; the band migrating ahead of BI but slower than IGF-II is the gold-labeled IGF-II.

Lane 8, 9: mono-NHS-Nanogold without IGF-II under the same reaction conditions.

Std: Standards: DBI (Di-BOC-insulin), BI (bovine insulin) and IGF-II.

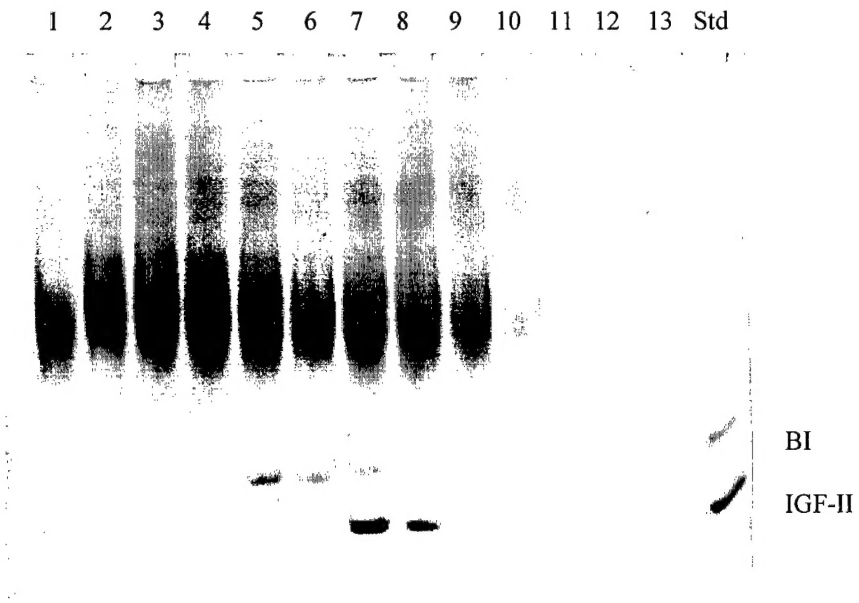

Figure 2 Acid-urea polyacrylamide gel electrophoresis of fractions from SEC-HPLC separation.

Lane 1: Reaction mixture from incubation of mono-NHS-Nanogold with IGF-II at 29-30° C for 43 hours.

Lanes 2 – 13: Fractions obtained by size exclusion chromatography. Fraction 6 and 7 were pooled for re-run.

Std : BI (bovine insulin) and IGF-II

Figure 3 Silver-stained reduced SDS-PAGE of purified IR-A showing the 135-KDa α subunit and the 95-KDa β subunit.

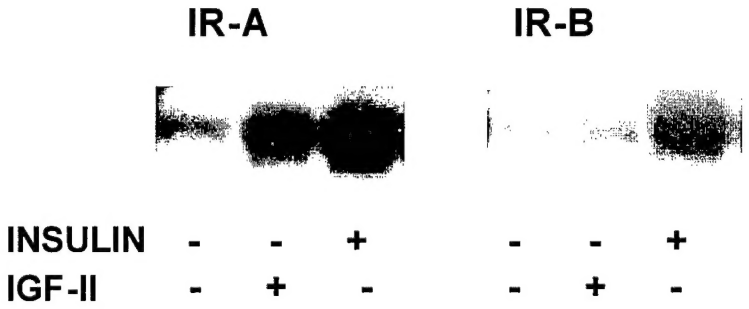

Figure 4 Western blot with anti-PY comparing IR-A and IR-B insulin receptor autophosphorylation, showing IGF-II stimulating IR-A but not IR-B as effectively as insulin.

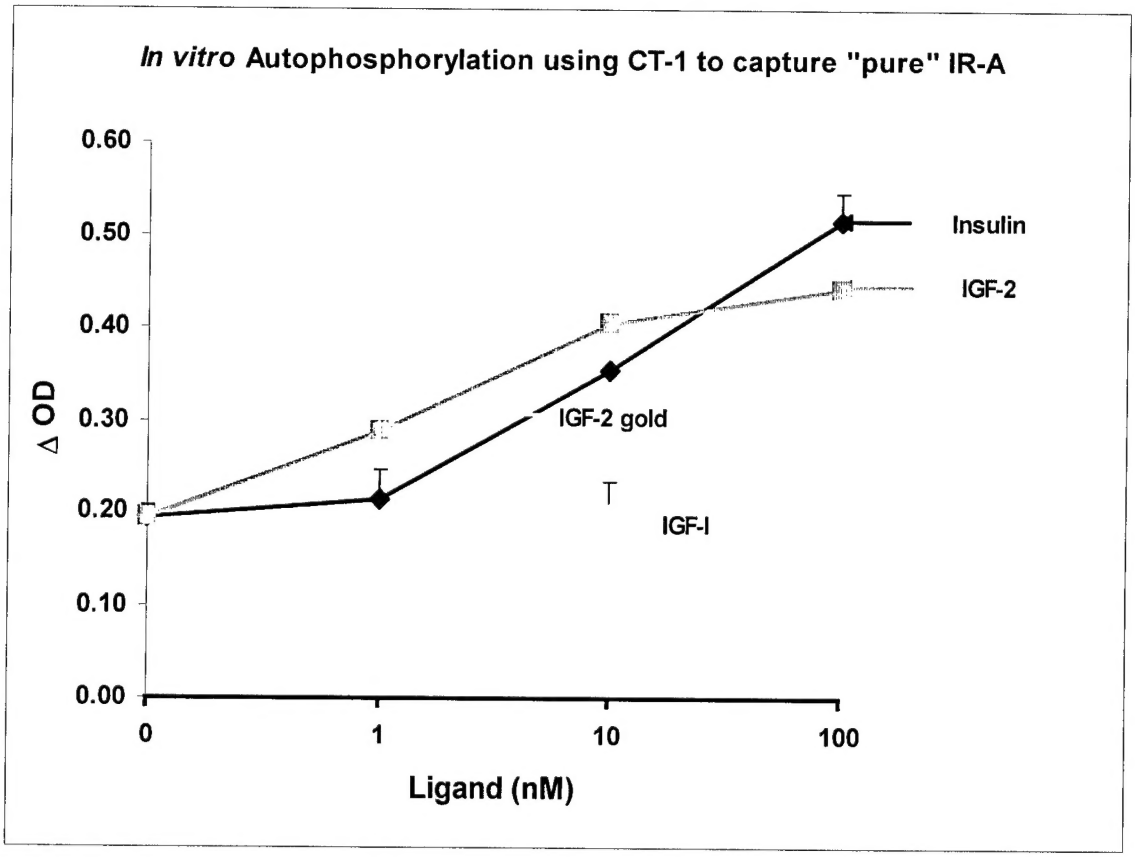

Figure 5 ELISA of stimulation of receptor (IR-A) autophosphorylation by insulin, IGF-I, IGF-II (IGF-2), and Nanogold-IGF-II (IGF-2 gold), showing that Nanogold-IGF-II was as active as unmodified IGF-II.